APPLIED MICROBIOLOGY, July 1970, p. 98-102

Vol. 20, No. 1
Printed in U.S.A.

Susceptibility of *Pseudomonas aeruginosa* to Gentamicin Sulfate In Vitro: Lack of Correlation Between Disc Diffusion and Broth Dilution Sensitivity Data

WALTER H. TRAUB

Departments of Pathology and Microbiology, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

Received for publication 7 April 1970

Seventy-eight of 420 clinical isolates of *Pseudomonas aeruginosa* yielded zones of inhibition of less than 12 mm in diameter around 10-μg discs of gentamicin sulfate when tested by the standardized Bauer-Kirby disc diffusion method. Of 153 strains chosen from these isolates, one strain (0.65%) required 25 μg of gentamicin per ml for inhibition; the remainder (99.35%) were inhibited by 6 μg/ml or less of the antibiotic. It is recommended that those isolates of *P. aeruginosa* that yield zones of inhibition less than 12 mm in diameter be disc susceptibility-tested once more; those isolates that give zones of inhibition of less than 12 mm upon repeated examination should then be subjected to the broth dilution test before they are designated as sensitive or resistant to gentamicin.

Recently, it was recommended (4) that organisms yielding zones of inhibition of 12 to 13 mm in diameter or more around gentamicin sulfate discs (10 μg) be designated as sensitive to this antibiotic when tested by the standardized technique of Bauer et al. (1). This recommendation was found to be generally valid with regard to our clinical isolates of *Enterobacteriaceae* and *Micrococcaceae*. However, difficulties were encountered with isolates of *Pseudomonas aeruginosa* and other pseudomonads. The present study was prompted by the finding that 78 of 420 clinical isolates (18.6%) of *P. aeruginosa* yielded zones of inhibition measuring less than 12 mm in diameter. It seemed highly unlikely that such a significant number of these isolates were resistant to the antibiotic.

MATERIALS AND METHODS

Bacteria. Strains of *P. aeruginosa* isolated from various clinical sources were identified by conventional criteria, among them the oxidase reaction, the production of pigments and of a metallic sheen, and the oxidative metabolism of carbohydrates. A strain of *Escherichia coli* of known antibiotic sensitivity served for control purposes.

Gentamicin sulfate. Nonsterile gentamicin sulfate powder (579 μg of activity per mg) was a gift from the Schering Corp., Union, N.J. (batch GMC-8-M-65-1), as were 10-μg gentamicin discs. The latter had been prepared by Difco Laboratories, Detroit, Mich. (batches 9AMW 206, 505807, and 505784) and the Baltimore Biological Laboratories, Baltimore, Md. (batches no. 9AMW10 and 9AMW13). The antibiotic powder was dissolved in sterile distilled water to yield 2,000 μg/ml, passed through 0.2-μm membrane filters (Millipore Corp., Bedford, Mass.), dispensed as 2-ml samples into sterile, small screw-capped vials, and frozen and kept stored at −15 C. The vials were never refrozen after thawing.

Media. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were purchased from Difco. The organisms under study were maintained on nutrient agar slants (Difco).

Sensitivity tests. Disc diffusion sensitivity tests were done in a manner identical to that of Bauer et al. (1). Broth dilution tests, utilizing serial $\log_2$ dilutions of gentamicin over the range of 50 to 0.025 μg/ml, were performed by using MHB and a bacterial inoculum standardized to yield 1.5×10^6 organisms/ml at 0 time. For this purpose, organisms in the exponential growth phase (pregrown for 6 hr at 35 C in MHB) were adjusted to McFarland $BaSO_4$ standard no. 0.5, the turbidity of which corresponds to that of 1.5×10^8 organisms/ml. The adjusted suspension of organisms was further diluted 50-fold in MHB (corresponding to 3×10^6 organisms/ml). Assay tubes received 1 ml of the respective double-strength dilution of antibiotic and 1 ml of bacterial inoculum. Control tubes received 1 ml of MHB and 1 ml of bacterial inoculum. The assay and control tubes were incubated at 35 C for 18 hr. The minimal inhibitory

concentration (MIC) of gentamicin sulfate was defined as the lowest concentration of antibiotic completely inhibiting growth as judged by visual inspection. The minimal bactericidal concentration (MBC) of the drug was determined through subculture of one 3-mm loopful from clear tubes to quarter sectors of 5% sheep blood-agar plates which were incubated at 35 C for 24 hr. The MBC was defined as the lowest concentration of gentamicin yielding no growth after subculture to blood-agar.

RESULTS

As stated above, 18.6% or 78 strains of the clinical isolates of *P. aeruginosa* proved "resistant" to gentamicin by the disc diffusion sensitivity method [6 mm (no zone of inhibition as discs measure 6 mm), 15 strains; zone of inhibition 7 mm, 2 strains; 8 mm, 4 strains; 9 mm, 8 strains; 10 mm, 26 strains; 11 mm, 23 strains], employing BBL discs (batch 9AMW10). A total of 22 strains yielded zones greater than 20 mm in diameter; most of these isolates were either "mucoid" or "brown-pigmented" strains of *P. aeruginosa*. The majority of strains (279 = 66.4%) gave zones of inhibition measuring 12 to 16 mm in diameter (zone of inhibition 12 mm, 57 strains; 13 mm, 61 strains; 14 mm, 50 strains; 15 mm, 65 strains; 16 mm, 46 strains). Forty-one strains gave zones of inhibition measuring 17 to 20 mm in diameter (17 mm, 18 strains; 18 mm, 10 strains; 19 mm, 9 strains; 20 mm, 4 strains). The control strain of *E. coli*, which was inhibited by 0.8 μg of gentamicin per ml, yielded zones of inhibition measuring 21 mm in diameter.

Of these 420 isolates of *P. aeruginosa*, 153 strains were examined simultaneously by both methods of sensitivity testing (Table 1). Only one strain was found to be resistant to gentamicin sulfate, requiring 25 μg of the antibiotic per ml for inhibition. The remaining strains (99.35%) were sensitive to 6 μg/ml or less of the antibiotic. Of these 153 strains, a total of 14 strains, including the sole resistant strain, yielded zones of inhibition less than 12 mm in diameter (BBL discs, batch 9AMW10); yet 13 of these strains were sensitive to the antibiotic as determined by the broth dilution technique, in that they were inhibited by 3 μg/ml or less of the antibiotic.

In a further series of experiments, 74 of 153 isolates were tested with both Difco and BBL discs (batches 9AMW206 and 9AMW10, respectively). Nine strains were "resistant" when tested with BBL discs, whereas 12 strains had to be designated as "resistant" in the case of Difco discs, in that the zones of inhibition obtained amounted to less than 12 mm in diameter. With 60 of the 74 isolates examined, Difco discs (batch 9AMW206) gave zones of inhibition that were 1 to 2 mm smaller in diameter than those obtained with discs manufactured by BBL (batch 9AMW10).

Eleven additional isolates of *P. aeruginosa* were exposed to five different batches of 10-μg gentamicin discs in an effort to detect any batch-to-batch variation among these discs. In addition, the MIC (24 and 48 hr of incubation at 35 C) and the MBC of gentamicin were determined simultaneously against these strains. A significant number of these strains that were fully susceptible to the antibiotic as revealed by the broth dilution technique yielded incompatible zones of inhibition (Table 2). Specifically, Difco discs of batch 9AMW206 yielded somewhat smaller zones of inhibition, whereas Difco discs of batches 505807 and 505784 produced zones slightly larger than those obtained with BBL discs. Generally, the 24- and 48-hr MIC values of gentamicin against these isolates coincided or varied by one twofold dilution, although in 2 of 11 cases the 48-hr MIC was found to be fourfold greater than the 24-hr MIC. The MBC of gentamicin against these isolates of *P. aeruginosa* usually was four- to eightfold higher than

TABLE 1. *Lack of correlation between minimal inhibitory concentrations and zones of inhibition obtained with gentamicin against 153 clinical isolates of Pseudomonas aeruginosa*

MIC (μg/ml)	1	2	0	1	4	6	11	24	40	26	16	12	4	4	2	153	Total
25						1										1	
12																0	
6								1								1	
3					1	2			2			1				6	
1.5		1				2	2	3	1							9	
0.8	1	1		1	1		4	6	11	5	1	2				33	
0.4					2	1	1	10	12	8	7	1				42	
0.2							4		10	9	8	4	4	1	2	42	
0.1								4	4	4		4		3		19	
0.05																0	
0.025																0	
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		

Diameter of inhibition zones (in mm)

TABLE 2. *Batch-to-batch variation of 10-μg gentamicin discs*

Isolate	Disc diffusion test (zones of inhibition in mm)					Broth dilution test[a]		
	BBL		Difco					
	9AMW13	9AMW10	9AMW206	505807	505784	24-hr MIC (μg/ml)	48-hr MIC (μg/ml)	MBC (μg/ml)
234	13	13	12	13	14	0.4	0.8	3
254	13	12	12	13	13	0.4	0.8	3
1648	12	12	11	13	13	0.8	0.8	3
1659	13	13	13	14	14	0.4	0.8	1.5
1979	10	10	9	11	11	0.4	1.5	3
1682	13	13	12	14	13	0.2	0.8	3
1651	12	12	11	13	13	0.8	0.8	6
147	12	12	11	13	13	0.4	0.8	3
360	13	13	12	14	14	0.4	0.8	1.5
345	13	14	14	15	15	0.2	0.2	0.8
368	14	13	14	15	14	0.2	0.4	1.5
Control *E. coli*	20	21	21	21	21	0.8	1.5	1.5

[a] MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

the respective 48-hr MIC, confirming the observations of others (2, 6, 7, 10).

It was found that gentamicin sulfate was as effective against exponentially growing as against stationary-phase *P. aeruginosa* organisms. As shown in Table 3, the MIC values obtained with exponentially growing cells (pregrown for 6 hr at 35 C) coincided with the MIC values of those organisms that had been in the stationary phase of growth (pregrown for 24 hr at 35 C) at 0 time (5).

It was of interest to determine the number of naturally occurring less susceptible variants in populations of gentamicin-sensitive isolates of *P. aeruginosa*, since Weinstein (9) had found that resistance to gentamicin developed slowly and not in a single-step fashion. For this purpose, the turbidity of 13 isolates in the exponential growth phase and the turbidity of the control *E. coli* were adjusted to that of McFarland $BaSO_4$ standard no. 0.5 (1.5×10^8 organisms/ml), and one 3-mm loopful (roughly 5×10^6 organisms) of the standardized suspensions was streaked to sectors of MHA plates into which serial twofold dilutions of gentamicin in MHB had been incorporated. The plates were read after 24 and 48 hr of incubation at 35 C. The least susceptible variants encountered, which required 48 hr of incubation to become readily visible as well-defined colonies, were subcultured to blood-agar, oxidase-characterized, and tested for their MIC and MBC by the broth dilution technique. The 24-hr MIC of gentamicin against these variants differed only from two- to eightfold from that against the parent strains (Table 4). After 48 hr of incubation, the MIC values of gentamicin were generally 16-fold higher. However, the MBC values obtained for these variants were markedly higher than those of the corresponding parent strains, although strain 1651 was exceptional in this regard. Four of the five variants tested with 10-μg gentamicin discs yielded no zone of inhibition.

TABLE 3. *Effectiveness of gentamicin against organisms in the exponential and stationary phases of growth*

Isolate	Organisms in exponential growth phase at 0 time		Organisms in stationary growth phase at 0 time	
	24-hr MIC (μg/ml)	48-hr MIC (μg/ml)	24-hr MIC (μg/ml)	48-hr MIC (μg/ml)
1731	0.4	0.8	0.4	0.8
612	0.4	0.8	0.4	0.8
1282	0.4	0.8	0.8	0.8
1536	0.8	0.8	0.8	0.8
1590	0.4	0.4	0.4	0.4
1676	0.8	0.8	0.8	1.5
1843	0.8	1.5	0.4	1.5
1894	1.5	1.5	1.5	1.5
Control *E. coli*	0.8	1.5	0.8	1.5

In another series of experiments, 3 of the above 13 isolates of *P. aeruginosa* and the control *E. coli* were grown in MHB for 24 hr; their turbidity was adjusted to that of a McFarland standard no. 10 (corresponding to 3×10^9 organisms/ml). A 3-mm loopful of each suspension as well as of 1:10 and 1:100 dilutions thereof were streaked to quarter sectors of MHA plates containing serial twofold dilutions of gentamicin; the sectors thus were seeded with approximately 10^8, 10^7, and 10^6 organisms, respectively. The plates were incubated for a total of 48 hr at 35 C, after which time the variants growing at the highest concentration of gentamicin were oxidase-tested and recorded (Table 5); each of the strains revealed a small number of variants that were less susceptible to gentamicin and which could be detected only when relatively large bacterial inocula were used, i.e., about 10^7 to 10^8 organisms.

In view of the findings of Garrod and Waterworth (3) and those of Washington et al. (8), namely that the activity of gentamicin was reduced in the presence of agar, five isolates of *P. aeruginosa* and the control *E. coli* (in the exponential growth phase) were examined with the broth dilution and the agar dilution methods

TABLE 4. *Naturally occurring less susceptible variants in populations of gentamicin-susceptible isolates of Pseudomonas aeruginosa*[a]

Isolate	Broth dilution test (parent isolate)		Growth (48 hr) of least susceptible variants[b]	Broth dilution sensitivity of least susceptible variants			Disc diffusion sensitivity of least susceptible variants	
	24-hr MIC (μg/ml)	MBC (μg/ml)		24-hr MIC (μg/ml)	48-hr MIC (μg/ml)	MBC (μg/ml)	BBL (9AMW13)	Difco (505784)
234	0.4	3	12(2)[c]					
254	0.4	3	25(1)	1.5	6	25	6[d]	6
1648	0.8	3	12(1)					
1659	0.4	1.5	12(6)					
1979	0.4	3	12(30)					
1682	0.2	3	12(3)					
1651	0.8	6	25(1)	1.5	3	6	15	15
147	0.4	3	50(1)	3	12	25	6	6
398	0.4	1.5	25(1)	1.5	6	12	6	6
360	0.4	1.5	50(1)	3	6	25	6	6
345	0.2	0.8	6(3)					
368	0.2	1.5	12(2)					
371	0.2	1.5	25(1)	1.5	3	6	6	6
Control *E. coli*	0.8	1.5	1.5(3)					

[a] A total of 5×10^6 organisms were streaked to sectors of gentamicin-MHA dilution plates.

[b] At highest concentration (micrograms per milliliter) of glutamicin on MHA.

[c] Numbers in parentheses indicate number of colonies of least susceptible variants obtained at highest concentration of gentamicin after 48 hr of incubation.

[d] Diameters (in millimeters) of zones of inhibition. Discs measured 6 mm in diameter; thus, a reading of 6 mm means no visible zone of inhibition.

TABLE 5. *Semiquantitation of less susceptible variants naturally occurring in gentamicin-susceptible populations of Pseudomonas aeruginosa*

Isolate	Growth (48 hr) of least susceptible variants at highest concn (μg/ml) of gentamicin in MHA		
	10^{6}[a]	10^{7}[a]	10^{8}[a]
147	25(1)[b]	12(8)	50(6)
360	6(>100)	50(1)	100(1)
371	12(1)	12(4)	25(11)
Control *E. coli*	0.8(6)	1.5(7)	3(10)

[a] Number of organisms streaked to sectors of gentamicin-MHA dilution plates.

[b] Numbers in parentheses indicate number of colonies of least susceptible variants obtained at highest concentration of gentamicin after 48 hr of incubation.

TABLE 6. *Comparison of minimal inhibitory concentrations (MIC) of gentamicin against Pseudomonas aeruginosa obtained with the agar and broth dilution methods*

Isolate	Broth dilution test			Agar dilution test			
				20 ml of MHA per plate, sectors seeded with		10 ml of MHA per plate, sectors seeded with	
	24-hr MIC	48-hr MIC	MBC	1.5×10^2 organisms	1.5×10^4 organisms	1.5×10^2 organisms	1.5×10^4 organisms
254	0.4[a]	0.8	3	6	6	6	6
1651	0.8	0.8	6	6	12	6	12
147	0.4	0.8	3	6	6	6	6
398	0.4	0.8	1.5	6	6	6	6
360	0.4	0.8	1.5	6	6	6	6
Control *E. coli*	0.8	1.5	1.5	0.4	0.8	0.8	0.8

[a] All values listed represent micrograms per milliliter of gentamicin.

of sensitivity testing. The broth dilution tests were performed as described above. Gentamicin in MHB was incorporated into MHA in serial twofold dilutions ranging from 100 to 0.1 μg/ml; plates (100 by 15 mm) received a final volume of 20 and 10 ml of medium containing a specified concentration of gentamicin, respectively. The two different volumes of agar were chosen to exclude varying thickness of MHA as a possible variable in agar dilution tests. The turbidity of each strain was adjusted to that of McFarland standard no. 0.5, and the standardized suspensions were further diluted 100- and 1,000-fold in MHB. Calibrated loops delivering 0.001 ml served to spot-inoculate the organisms; thus, the sectors were seeded with an inoculum of 1.5×10^2 and 1.5×10^3 organisms, respectively. The plates were incubated for 18 hr at 35 C. The agar dilution MIC values were essentially identical for both bacterial inocula; furthermore, the MIC values obtained were identical regardless of whether the dilution plates had received a 20- or 10-ml final volume of MHA (Table 6). However, the agar dilution MIC values were 8- to 16-fold higher than those obtained with the broth dilution technique, and, with the exception of strain 1651, 2- to 4-fold higher than the corresponding broth dilution MBC values obtained. On the other hand, the control strain of *E. coli* was inhibited by gentamicin to the same extent by both dilution methods of sensitivity testing.

DISCUSSION

The Schering Corp., following the lead of Kirby and Standiford (4), recommended that a zone of 13 mm or greater around 10-μg gentamicin discs is indicative of sensitivity of isolates to this antibiotic. Furthermore, it was specifically recommended that those pseudomonads that appeared to be resistant by the disc diffusion test be subjected to the broth dilution sensitivity test. Our results indicate the need for broth dilution tests for all those strains of *P. aeruginosa* that yield zones of inhibition of less than 12 mm in diameter with 10-μg gentamicin sulfate discs, as judged from the standardized technique of Bauer et al. (1). It should be stressed that no difficulties of the above nature were encountered with staphylococcal and enterobacterial isolates; an exception were 30 isolates of a promptly (overnight) lactose-fermenting strain of *Proteus rettgeri* which was found to be resistant to all antibiotics and chemotherapeutic agents tested except methenamine mandelate (W. H. Traub, Experientia, *in press*); this strain, which was inhibited by 30 μg of gentamicin per ml in MHB, yielded zones of inhibition varying from 11 to 13 mm in diameter.

Based on the findings of this study, the following procedure was adopted in our clinical laboratory to determine the susceptibility of isolates to gentamicin sulfate. Staphylococci and *Enterobacteriaceae* are designated as sensitive to gentamicin if they yield zones of inhibition measuring 15 mm or more in diameter by the technique of Bauer et al. (1). Any isolate of *P. aeruginosa* that is found to yield an inhibition zone of less than 12 mm is disc-diffusion tested once more, as are those staphylococcal or enterobacterial isolates that yield zones of less than 15 mm in diameter. Those strains of *P. aeruginosa* that give zones of less than 12 mm upon repeated testing are subjected to the broth dilution sensitivity test before they are designated as sensitive or resistant to the antibiotic. The same applies for those staphylococcal and enterobacterial isolates that twice had yielded zones of inhibition of less than 15 mm in diameter.

Disc diffusion susceptibility results (10-μg gentamicin discs, BBL batch 9AMW13 and Difco batch 505784) pertaining to an additional 162 clinical isolates of *P. aeruginosa* are as follows. With the revised zone criterion, only 6 (3.7%) of these 162 isolates had to be broth dilution sensitivity tested [6 mm (no visible zone of inhibition as discs measured 6 mm), one strain; zone of inhibition 8 mm, one strain; 10 mm, one strain; 11 mm, three strains]; their MIC values ranged from 0.2 to 1.5 μg/ml. Had a zone of 13 mm in diameter been selected as the breakpoint, 31 (19.1%) of the isolates would have had to be examined with the broth dilution technique. A total of 156 (96.3%) sensitive strains were detected with the disc diffusion susceptibility test (zone of inhibition 12 mm, 25 strains; 13 mm, 28 strains; 14 mm, 36 strains; 15 mm, 25 strains; 16 mm 20 strains; 17 mm, 6 strains; 18 mm, 3 strains; 19 mm, 1 strain; 20 mm, 3 strains; >20 mm, 9 strains).

Of particular interest was the finding that several susceptible isolates of *P. aeruginosa* yielded small numbers of variants that proved less susceptible to gentamicin. These variants, of course, could not be detected by the agar dilution method, since the bacterial inocula customarily employed are too small. Broth dilution tests also would fail to detect these variants because of the relatively small bacterial inoculum used (1.5×10^6 organisms/ml at 0 time in our laboratory); incubation of assay tubes beyond 48 hr at 35 C did not result in higher MIC or MBC values.

The finding that gentamicin was less effective in agar than in broth against isolates of *P. aeruginosa* confirms the observations of Garrod and Waterworth (3); these authors found that increased concentrations of magnesium ions in agar or broth progressively diminished the activity of gentamicin against *P. aeruginosa* but not against *E. coli*.

ACKNOWLEDGMENT

This study was supported by a grant from the United Medical Research Foundation of North Carolina.

I thank Pat Reich for competent assistance.

LITERATURE CITED

1. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Amer. J. Clin. Pathol. 45:493–496.
2. Bulger, R. J., S. Sidell, and W. M. M. Kirby. 1963. Laboratory and clinical studies of gentamicin. A new broad-spectrum antibiotic. Ann. Int. Med. 59:593–604.
3. Garrod, L. P., and P. M. Waterworth. 1969. Effect of medium composition on the apparent sensitivity of *Pseudomonas aeruginosa* to gentamicin. J. Clin. Pathol. 22:534–538.
4. Kirby, W. M. M., and H. C. Standiford. 1969. Gentamicin: *in vitro* studies. J. Infec. Dis. 119:361–363.
5. Rubenis, M., V. M. Kozij, and G. G. Jackson. 1964. Laboratory studies on gentamicin. Antimicrob. Ag. Chemother.—1963, p. 153–156.
6. Sander, J., and C. Blasius. 1968. Untersuchungen über die Wirksamkeit von Gentamycin gegen bestimmte gramnegative Staebchenbakterien. Deut. Med. Wochenschr. 93:1401–1404.
7. Waitz, J. A., and M. J. Weinstein. 1969. Recent microbiological studies with gentamicin. J. Infec. Dis. 119:355–360.
8. Washington, M. J., II, R. E. Ritts, Jr., and W. J. Martin. 1970. *In vitro* susceptibility of gram-negative bacilli to gentamicin. Mayo Clinic Proc. 45:146–149.
9. Weinstein, M. J. 1967. Experimental background and bacteriology of gentamicin, p. 9–18. Gentamicin, 1st Int. Symp., Paris (Schwabe Basel).
10. Weinstein, M. J., G. M. Luedemann, E. M. Oden, and G. H. Wagman. 1964. Gentamicin, a new broad-spectrum antibiotic complex. Antimicrob. Ag. Chemother.—1963, p. 1–7.